Reovirus infection is regulated by NPC1 and endosomal cholesterol homeostasis

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43 **Running title:** Reovirus infection is regulated by NPC1 and endosomal cholesterol
44 homeostasis

Abstract

Cholesterol homeostasis is required for the replication of many viruses, including Ebola virus, hepatitis C virus, and human immunodeficiency virus-1. Niemann-Pick C1 (NPC1) is an endosomal-lysosomal membrane protein involved in cholesterol trafficking from late endosomes and lysosomes to the endoplasmic reticulum. We identified NPC1 in CRISPR and RNA interference screens as a putative host factor for infection by mammalian orthoreovirus (reovirus). Following internalization via clathrin-mediated endocytosis, the reovirus outer capsid is proteolytically removed, the endosomal membrane is disrupted, and the viral core is released into the cytoplasm where viral transcription, genome replication, and assembly take place. We found that reovirus infection is significantly impaired in cells lacking NPC1, but infection is restored by treatment of cells with hydroxypropyl- β -cyclodextrin, which binds and solubilizes cholesterol. Absence of NPC1 did not dampen infection by infectious subvirion particles, which are reovirus disassembly intermediates that bypass the endocytic pathway for infection of target cells. NPC1 is not required for reovirus attachment to the plasma membrane, internalization into cells, or uncoating within endosomes. Instead, NPC1 is required for delivery of transcriptionally active reovirus core particles into the cytoplasm. These findings suggest that cholesterol homeostasis, ensured by NPC1 transport activity, is required for reovirus penetration into the cytoplasm, pointing to a new function for NPC1 and cholesterol homeostasis in viral infection.

65 **Author summary**

66 Genetic screens are useful strategies to identify host factors required for viral infection.
 67 NPC1 was identified in independent CRISPR and RNA interference screens as a
 68 putative host factor required for reovirus replication. We discovered that NPC1-
 69 mediated cholesterol transport is dispensable for reovirus attachment, internalization,
 70 and disassembly but required for penetration of the viral disassembly intermediate from
 71 late endosomes into the cytoplasm. These findings pinpoint an essential function for
 72 cholesterol in the entry of reovirus and raise the possibility that cholesterol homeostasis
 73 regulates the entry of other viruses that penetrate late endosomes to initiate replication.

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INTRODUCTION

Viral replication is dependent on cellular proteins and pathways for entry, transport, and release of the viral genome to sites of replication in the cell. Viral attachment to host cells occurs by interactions with cell-surface proteins, lipids, and carbohydrate moieties at the plasma membrane and often triggers virus uptake by receptor-mediated endocytosis (1-7). Viruses that traverse through endosomes must escape the endosomal compartment and release their genomes at sites of replication to initiate productive infection. Enveloped viruses generally accomplish endosomal escape using mechanisms involving receptor- or pH-mediated fusion of the viral envelope and endosomal membrane (6, 8-10). In contrast, nonenveloped viruses penetrate endosomal membranes by establishing small membrane pores or large membrane disruptions (9, 11-13). While both enveloped and nonenveloped viruses depend on conformational changes of viral structural proteins to escape endosomes, mechanisms underlying nonenveloped virus membrane penetration are not well understood (6).

Mammalian orthoreoviruses (reoviruses) are nonenveloped icosahedral viruses that infect a broad range of mammalian hosts. Reovirus infections are usually asymptomatic in humans, but these viruses have been implicated in development of celiac disease (14). Reovirus virions include two protein shells, the outer capsid, composed primarily of $\mu 1$ - $\sigma 3$ heterohexamers, and core (15-17). The core contains 10 segments of double-stranded (ds) RNA, which are classified by size into three large (L), three medium (M), and four small (S) segments (17). Following receptor-mediated endocytosis, the reovirus outer capsid undergoes a series of conformational changes and disassembly events required for release of transcriptionally active cores into the cytoplasm (18, 19).

99 Within late endosomes, acid-dependent cathepsin proteases catalyze proteolysis
100 of the viral outer-capsid protein $\sigma 3$ and cleavage of the membrane-penetration protein
101 $\mu 1$ to δ and ϕ , resulting in formation of metastable intermediates termed infectious
102 subviral particles (ISVPs) (20-24). Endosomal lipid composition induces ISVPs to
103 undergo additional conformational changes resulting in exposure of hydrophobic
104 domains of δ , release of pore-forming fragment $\mu 1N$, and formation of ISVP*s (25, 26).
105 Release of $\mu 1N$ during ISVP-to-ISVP* conversion leads to endosomal penetration and
106 liberation of the viral core into the cytoplasm where infection progresses (27-31).
107 Although some essential viral and host factors required for reovirus penetration of the
108 endosome are known, the process is still not well understood.

109 In this study, we used CRISPR and RNA interference screens to discover that
110 Niemann Pick C1 (NPC1), an endolysosomal transmembrane protein that mediates
111 cholesterol egress from late endosomes for redistribution to cellular membranes (32-
112 34), is required for reovirus infection. We found that genetic ablation of NPC1 in human
113 brain microvascular endothelial cells (HBMECs) diminishes reovirus infection by virions
114 but not by ISVPs, suggesting that NPC1 is required for steps that differ between virions
115 and ISVPs. Treatment of NPC1-null HBMECs with hydroxypropyl-beta-cyclodextrin
116 (H β CD), a macrocycle that binds and solubilizes cholesterol, restored infectivity by
117 reovirus virions, suggesting that endosomal cholesterol homeostasis contributes to
118 efficient reovirus entry. While NPC1 is not required for viral attachment to the plasma
119 membrane, internalization, or uncoating within endosomes, we found that NPC1 is
120 required for efficient release of reovirus cores from endosomes into the cytoplasm.
121 Together, these findings suggest that cholesterol homeostasis, mediated by NPC1
122 cholesterol transport activity, is essential for reovirus cell entry and penetration into the
123 cytoplasm.

RESULTS

CRISPR/Cas-9 and siRNA screens for host factors required for reovirus infection identify NPC1

To discover host factors required for reovirus infection, we conducted genome-wide CRISPR/Cas-9 and siRNA-based cell-survival screens. The CRISPR/Cas-9 screen was conducted using BV2 mouse microglial cells with the murine Asiago sgRNA library targeting over 20,000 genes. BV2 CRISPR cell libraries were infected with reovirus strains type 1 Lang (T1L) and type 3 Dearing (T3D) and cultured for nine days prior to isolation of genomic DNA (gDNA) from surviving cells and deep sequencing. STARS analysis was conducted to identify enriched CRISPR gRNAs within the surviving cell population (Fig. 1A and Table S1). The siRNA screen was conducted using HeLa S3 cells transfected with the ON-TARGET plus siRNA whole genome library targeting over 18,000 genes (35). Transfected cells were infected with reovirus strain T3SA+ and scored for viability using an ATP-dependent luminescence assay. T3SA+ contains nine genes from T1L and the S1 gene from strain T3C44-MA (36). T3SA+ binds all known reovirus receptors and is cytolytic. Robust Z scores (median absolute deviation) were calculated for each sample (Fig. 1B and Table S2).

Key genes and pathways essential for reovirus replication were defined by comparing the CRISPR/Cas-9 and siRNA screen lists using STRING-db (Fig. 1C). In the CRISPR/Cas-9 screen, four functional pathways defined by Gene Ontology (GO) terms were common to both T1L and T3D, including sialic acid biosynthesis and metabolism (Fig. 1D). Sialic acid is a reovirus attachment factor, and genes involved in sialic acid biosynthesis and metabolism, including *Slc35a1*, are required for T3SA+ replication in BV2 cells (37). These data provide confidence that the target genes identified in the CRISPR/Cas-9 screen represent biologically significant candidates. We

also compared KEGG pathways identified in the CRISPR/Cas-9 and siRNA screens to increase the likelihood of significant gene targets. Ribosome and lysosome pathways were the only pathways common to both screens (Fig. 1E). Lysosomal genes include *Ctsl*, *Neu1*, and *Npc1*. *Ctsl* encodes cathepsin L, which is required for cleavage of the reovirus outer capsid to form ISVPs (22). *Neu1* encodes neuraminidase, a lysosomal sialidase that cleaves sialic acid linkages required for reovirus infectivity (38). *Npc1* encodes NPC1, a cholesterol transporter that resides in the limiting membrane of endosomes and lysosomes (33, 34).

Engineering and characterization of HBMECs with CRISPR-targeted *Npc1*

Based on the function of NPC1 in cell entry and replication of other viruses (39) and its identification in both CRISPR and siRNA screens, we evaluated a potential role for NPC1 in reovirus replication. Human brain microvascular endothelial cells (HBMECs) are susceptible to reovirus infection (40) and amenable to CRISPR/Cas-9 gene editing (41). To facilitate these studies, we used CRISPR/Cas-9 gene editing to engineer a clonal HBMEC cell line lacking the *NPC1* gene (KO cells). The NPC1 KO cells were complemented by stable transfection of a functional NPC1 allele (KO+ cells).

The newly engineered NPC1 KO and KO+ cell lines were characterized for NPC1 expression and cholesterol distribution relative to wild-type (WT) HBMECs. Expression of NPC1 in WT, KO, and KO+ cells was tested using immunoblotting. As anticipated, NPC1 expression in KO cells was abrogated relative to WT and KO+ cells (Fig. S1A). There was an observable increase in NPC1 expression in KO+ cells compared with WT cells (Fig. S1B), but the difference was not statistically significant. In the absence of functional NPC1, cholesterol reorganizes from a homogeneous distribution to accumulate in endosomal compartments (32, 33). To define the

174 distribution of cholesterol in NPC1-null HBMECs, we used fluorescent filipin III to label
175 cholesterol in fixed cells and imaged cholesterol distribution using fluorescence
176 microscopy (Fig. S1C). Cholesterol distribution was homogeneous in WT (Fig. S1C,
177 left) and KO+ cells (Fig. S1C, right). However, cholesterol accumulated around the
178 nucleus in KO cells (Fig. S1C, center) in a pattern consistent with the distribution of
179 endosomes (Fig. S1D), confirming the absence of functional NPC1. Thus, KO cells
180 display the expected phenotype of altered cholesterol distribution when NPC1-
181 dependent cholesterol transport is disrupted. Furthermore, complementing NPC1
182 expression in KO cells restores the normal distribution of cholesterol, demonstrating
183 that the observed phenotype is specific for NPC1 expression.

184

185 **Reovirus infection by virions but not by ISVPs is impaired in NPC1 KO cells**

186 ISVPs prepared by treatment of virions *in vitro* with intestinal or endosomal proteases
187 bind to reovirus receptors and enter target cells by direct penetration of the plasma
188 membrane and bypass requirements for internalization into the endocytic compartment
189 and acid-dependent proteolysis (21, 22, 42). To determine whether NPC1 is required
190 for reovirus replication, and further whether NPC1 mediates a step in the infectious
191 cycle that differs between virions and ISVPs, we adsorbed WT, KO, and KO+ cells with
192 reovirus strain T1L M1 P208S virions or ISVPs. Reovirus T1L M1-P208S contains a
193 point mutation in the M1 gene that causes viral factories to have a globular morphology
194 similar to the morphology of factories formed by reovirus T3D (43), which renders
195 infected cells easier to detect. Infected cells were visualized by immunofluorescence
196 (IF) staining for reovirus antigen at 18 h post-adsorption (Fig. 2). Following adsorption
197 with reovirus virions, the number of infected KO cells was reduced by approximately
198 50% relative to infected WT and KO+ cells (Fig. 2A). A similar reduction in the number

199 of infected KO cells relative to WT and KO+ cells was observed when WT, KO, and
 200 KO+ cells were adsorbed with T1L, T3D, and T3SA+ virions, the reovirus strains used
 201 in the CRISPR/Cas9 and siRNA screens (Fig. S2). In contrast, no significant differences
 202 in numbers of infected cells were observed following adsorption of WT, KO, and KO+
 203 cells with ISVPs (Fig. 2B). Viral progeny production and release was determined by
 204 quantifying viral titers in cell lysates and supernatants at 0, 24, and 48 h following
 205 adsorption of WT, KO, and KO+ cells with virions or ISVPs. Following infection by
 206 virions, viral titers in lysates and supernatants of KO cells were 10- to 100-fold less than
 207 those in WT and KO+ cells (Fig. 2C and E). In contrast, following infection by ISVPs,
 208 viral titers in lysates and supernatants of all three cell types were comparable (Fig. 2D
 209 and F). Together, these results suggest that NPC1 is required for reovirus infection and
 210 functions at a step in the infectious cycle that differs between virions and ISVPs.

211

212 **NPC1 is not required for reovirus attachment, internalization, or uncoating**

213 Reovirus entry can be divided into four main stages: viral binding to cell-surface
 214 receptors, viral internalization by endocytosis, proteolytic removal of the viral outer
 215 capsid, and penetration of the core from late endosomes into the cytosol (19). We
 216 characterized NPC1 KO cells for the capacity to support each step of the reovirus entry
 217 pathway to define the function of NPC1 in reovirus infection. To determine whether
 218 NPC1 is required for reovirus attachment to target cells, we quantified viral binding
 219 using flow cytometry. The quantity of virus bound to the surface of all three cell types
 220 was comparable, and no statistically significant differences were observed (Fig. 3A).
 221 These data suggest that reovirus attachment to cells is not dependent on expression of
 222 NPC1.

223 To determine whether NPC1 is required for reovirus to access the endocytic
224 pathway of target cells, WT, KO, and KO+ cells were adsorbed with fluorescently-
225 labeled reovirus particles and monitored for reovirus uptake using live-cell imaging. We
226 found that the kinetics of reovirus internalization into WT, KO, and KO+ cells were
227 comparable. High-magnification videos (Videos 1, 2, and 3) along with static images
228 obtained at different intervals (Fig. 3B) demonstrate that attached reovirus particles
229 internalize slowly in the first ~ 0 - 10 min post-adsorption. During this time, reovirus
230 particles remain in the periphery, with a few particles coalescing to form large
231 fluorescent puncta. Convergence of immunofluorescent signals suggests co-transport
232 of multiple viral particles in the same endocytic compartment, similar to that observed
233 during reovirus entry into neurons (44). After ~ 15 min post-adsorption, we observed
234 rapid recruitment of almost every fluorescent puncta to the perinuclear region.

235 To more precisely define the movement of reovirus virions during entry, we
236 analyzed the trajectories of individual fluorescent virions in Videos 1, 2, and 3 over 36
237 min using the Spot detector plugin function from Icy software. Trajectory colors change
238 over time in which each color corresponds to an interval of ~ 7.5 min in the time-lapse
239 videos (Videos 4, 5, and 6). Analysis of the time-dependent trajectories confirms
240 observations made in the live-imaging videos. Thus, video-microscopic analysis
241 demonstrates that reovirus virions are internalized rapidly into HBMECs and that virion
242 uptake into the endocytic pathway is not impaired in the absence of NPC1.

243 Following internalization of reovirus virions, acid-dependent cathepsin proteases
244 in late endosomes catalyze disassembly. During disassembly, proteolytic cleavage of
245 the outermost capsid protein, $\sigma 3$, exposes the membrane-penetration protein, $\mu 1$, which
246 is subsequently cleaved to form a variety of intermediates that lead to penetration of the
247 core particle into the cytoplasm (20-24, 27-30). Cells lacking NPC1 have increased

248 endosomal pH and decreased cathepsin activity (45), which could impair reovirus
249 uncoating. To determine whether NPC1 is required for reovirus disassembly, we
250 defined the kinetics of reovirus outer-capsid proteolysis in WT, KO, and KO+ cells by
251 following the formation of the δ cleavage fragment of the μ 1 protein. Cells were
252 adsorbed with reovirus virions, and viral proteins in cell lysates were visualized by
253 immunoblotting at 0, 1, 2, and 3 h post-adsorption using a reovirus-specific antiserum.
254 No significant differences in the kinetics of μ 1 proteolysis were observed, with an initial
255 δ cleavage product detected 2 h after adsorption in WT, KO, and KO+ cells (Fig. 3C).
256 These data suggest that the cathepsins that catalyze reovirus disassembly are not
257 impaired in NPC1 KO HBMECs. Collectively, these results demonstrate that NPC1 is
258 not required for reovirus receptor binding, internalization, or disassembly.

259

260 **Escape of reovirus cores from endosomes is impaired in cells lacking NPC1**

261 To determine whether NPC1 is required for escape of reovirus cores into the cytoplasm
262 following disassembly in the endocytic compartment, we imaged cores in fixed cells by
263 IF. Cells were adsorbed with fluorescently labeled reovirus virions and incubated in the
264 presence of cycloheximide for 8 h post-adsorption to inhibit synthesis of new viral
265 proteins and thus ensure detection of proteins from infecting viral particles. Cells were
266 stained with a CD-63-specific antibody to label endosomes and an antiserum specific
267 for reovirus cores and imaged using confocal microscopy. Small puncta consistent with
268 reovirus cores were observed in WT and KO+ cells, while in KO cells, cores appeared
269 to accumulate in larger puncta corresponding to endosomes (Fig. 4A). The distribution
270 of virions, cores, and endosomes was determined to quantify the extent of
271 colocalization. The results demonstrate frequent colocalization of cores and endosomes
272 in KO cells (Manders coefficient [Mc]: ~ 0.7), while there was much less colocalization

of cores and endosomes in WT and KO+ cells (Mc: ~ 0.3) (Fig. 4B). Colocalization of virions and cores also was more frequent in KO cells (Mc: ~ 0.45) than in WT (Mc: ~0.15) or KO+ (Mc: ~0.2) cells, whereas colocalization of virions and endosomes was comparable in all cell types (Mc: ~ 0.6). These data suggest that cores escape from endosomes more efficiently in the presence of NPC1.

To complement the imaging experiments, we quantified newly synthesized viral s4 mRNA using RT-qPCR. WT, KO, and KO+ cells were adsorbed with reovirus, RNA was isolated, and s4 transcripts were quantified at 0, 6, 12, and 24 h post-adsorption. We observed a statistically significant increase in total s4 RNA in WT and KO+ cells at 12 and 24 h post-adsorption relative to KO cells (Fig. 5). Together, these results suggest that NPC1 is required for release of transcriptionally active reovirus cores from endosomes into the cytoplasm.

Cholesterol homeostasis is required for reovirus entry

We thought it possible that NPC1 could serve as an endosomal receptor for reovirus and interact with one or more viral capsid proteins to enable core delivery into the cytoplasm, analogous to the function of NPC1 in Ebola virus infection (46, 47). Alternatively, NPC1 might be required to maintain an endosomal environment with appropriate cholesterol levels to allow cores to penetrate endosomes. To distinguish between these possibilities, we tested whether hydroxypropyl- β -cyclodextrin (H β CD), a cyclic oligosaccharide that triggers cholesterol release from the endo-lysosomal compartment (48, 49) and has been used to treat persons with Niemann-Pick disease type C (50, 51), for the capacity to overcome the effects of NPC1 deficiency on reovirus infection. To determine whether H β CD treatment redistributes cholesterol from endosomal membranes to a homogeneous distribution in the absence of NPC1, NPC1

298 KO HBMECs were treated with 1 mM H β CD, a non-toxic concentration (Fig. S3A), or
 299 PBS for 48 h prior to staining for the filipin III complex. Cells displaying cholesterol
 300 accumulation were distinguished from those with widely distributed cholesterol by
 301 quantifying the mean fluorescence intensity (MFI) of filipin III complex staining. Using
 302 this approach, an increase in MFI correlates with an increase in cholesterol
 303 accumulation. After H β CD treatment, KO cells displayed a significant redistribution of
 304 cholesterol, reducing its accumulation in endosomes and enhancing its distribution
 305 broadly throughout the cell, correlating with a statistically significant decrease in MFI
 306 (Fig. S3B,C). These data demonstrate that H β CD treatment promotes cholesterol efflux
 307 in KO cells, resulting in a cholesterol-distribution phenotype comparable to WT and
 308 KO+ cells (Fig. S3C).

309 Once we observed that H β CD treatment effectively redistributes cholesterol in
 310 KO cells and, thus, functionally complements NPC1 deficiency, we tested whether the
 311 reovirus entry defect in KO cells is due to the absence of NPC1 or impaired cholesterol
 312 homeostasis. WT, KO, and KO+ cells were pre-treated with 1 mM H β CD or PBS for 24
 313 h, adsorbed with reovirus virions or ISVPs, and scored for reovirus infection by
 314 immunostaining. Remarkably, H β CD treatment rescued infection of KO cells by reovirus
 315 virions (Fig. 6) but did not appreciably affect infection of WT or KO+ cells. H β CD
 316 treatment also did not affect infection of WT, KO, or KO+ cells by ISVPs. These data
 317 demonstrate that endosomal cholesterol homeostasis regulates reovirus entry by
 318 enhancing penetration of reovirus core particles into the cytoplasm.

DISCUSSION

In this study, we identified NPC1 as a putative host factor required for reovirus infection using genome-wide CRISPR/Cas9 and siRNA-based cell-survival screens. NPC1 is an endolysosomal cholesterol transporter that mediates cholesterol homeostasis (32-34). Disruption of NPC1 results in cholesterol accumulation in late endosomes (Sup. Fig. 2C) and leads to Niemann-Pick disease type C, an autosomal-recessive neurodegenerative disorder (32). Early steps in reovirus infection, including receptor binding, acid-dependent proteolytic disassembly, and ISVP-to-ISVP* conversion have been well characterized (19). However, penetration of endosomal membranes and release of viral cores into the cytoplasm are poorly understood processes. We used CRISPR/Cas9 gene-targeted HBMECs lacking NPC1 expression to study the function of NPC1 in reovirus infection. We discovered that NPC1 is dispensable for viral binding to cell-surface receptors (Fig. 3A), internalization of viral particles (Fig. 3B), and disassembly of the viral outer capsid (Fig. 3C). However, NPC1 is required for efficient penetration of reovirus cores into the cytoplasm (Fig. 4). Treatment with H β CD reduces cholesterol accumulation in endosomes (Sup. Fig. 3B and 3C) and restores reovirus infectivity in NPC1 KO cells (Fig. 6). These findings suggest that regulation of cholesterol in endosomal compartments is essential for reovirus entry into host cells.

NPC1 is required for the replication of several enveloped viruses. The filoviruses Ebola virus and Marburg virus use NPC1 as an intracellular receptor (46, 47). NPC1 also functions in enveloped virus replication by maintaining cholesterol homeostasis. Disruption of cholesterol homeostasis by inhibiting NPC1 prevents entry and replication of dengue virus (52) and African swine fever virus (53) and impairs exosome-dependent release of hepatitis C virus (54). Additionally, NPC1 has been implicated in cell entry of quasi-enveloped forms of hepatitis A virus and hepatitis E virus (55, 56). However,

344 NPC1 had not been previously appreciated to function in the replication of a
345 nonenveloped virus.

346 We found that reovirus binding, internalization, and uncoating do not require
347 NPC1, suggesting that NPC1 does not function as an intracellular receptor for reovirus.
348 Instead, we found that cholesterol accumulation in the endocytic pathway diminishes
349 the efficiency of reovirus core release into the cytoplasm. Using confocal microscopy,
350 we visualized and quantified the distribution of fluoresceinated reovirus virions, reovirus
351 cores, and late endosomes in infected cells (Fig. 4). Reovirus cores accumulate in the
352 lumen of late endosomes in KO cells (Fig. 4A), while virions distribute to endosomes
353 comparably in WT, KO, and KO+ (Fig. 4B). These findings suggest that cores do not
354 escape from endosomes efficiently in the absence of NPC1. RNA synthesis, which
355 occurs in the cytoplasm following release of cores from late endosomes, also was
356 reduced in KO cells relative to WT and KO+ cells (Fig. 5), providing evidence that core
357 escape from endosomes is required for initiation of transcription. It is not apparent how
358 cholesterol accumulation in KO cells blocks core release from late endosomes.

359 In Niemann-Pick disease type C, disruption of cholesterol homeostasis causes
360 changes in lipid composition of endosomal membranes (57, 58), inverting the ratio of
361 phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE). The change in PC:PE
362 ratio may alter mechanical properties of endosomal membranes by inhibiting intra-
363 endosomal membrane dynamics to favor negative curvature (57, 59). Membrane
364 composition and dynamics can influence viral entry. Negative membrane curvature
365 induced by addition of PE or the action of interferon-induced transmembrane protein 3
366 (IFITM3) impairs adenovirus protein VI-mediated membrane disruption (60) and
367 enveloped virus fusion (61), respectively. Although reovirus virions are nonenveloped,
368 entry of reovirus into cells also is inhibited by IFITM3 (62). Many nonenveloped viruses

use membrane-modifying proteins with the capacity to interact, destabilize, and disrupt membranes to mediate genome release into the cytoplasm (12, 63). However, the role of specific lipids in these processes is not well defined.

During reovirus entry, ISVP-to-ISVP* conversion leads to release of myristoylated μ 1N, which interacts with late endosomal membranes to facilitate release of cores into the cytoplasm (20-24). PE and PC concentrations in liposomes influence the efficiency of ISVP-to-ISVP* conversion (25). Therefore, it is possible that changes in membrane fluidity, width, or curvature caused by inversion of endosomal membrane PC:PE ratio in NPC1 KO cells impedes membrane insertion of μ 1N or formation and expansion of the penetration pore. Additionally, accumulation of cholesterol within the endosomal compartment of NPC1 KO cells could limit recruitment of ISVP*s to membrane-inserted μ 1N and the subsequent penetration of reovirus cores. Within the *Reoviridae* family, bluetongue virus (BTV) outer-capsid protein VP5 penetrates late endosomal membranes enriched in phospholipid lysobisphosphatidic acid (LBPA), which is dependent on the anionic charge and membrane fluidic properties of LBPA (64). LBPA-enriched late endosomes also are required for efficient rotavirus entry (65). Our data demonstrating the importance of cholesterol homeostasis in reovirus entry, along with the role of LBPA in BTV and rotavirus entry, suggest that the lipid composition of late endosomes influences nonenveloped virus entry and illuminate a potential new target for antiviral therapy.

Our findings parallel those of a companion study indicating a function for WD repeat-containing protein 81 (WDR81) in reovirus entry (66). WDR81 was identified in a CRISPR/Cas9 cell-survival screen using mouse embryo fibroblasts and found to be required for a step in reovirus entry that follows ISVP formation. WDR81 is required for the maturation of late endosomes by modulating levels of phosphatidylinositol 3-

phosphate (67). These findings, coupled with our studies of NPC1, suggest that ISVPs formed in an altered endocytic compartment of cells lacking either WDR81 or NPC1 cannot launch replication, whereas ISVPs adsorbed to the surface of such cells can. We think that alterations in cholesterol distribution might govern this difference in ISVP behavior.

Cholesterol accumulation due to NPC1 dysfunction also can lead to alterations in the distribution of host proteins, such as annexin A2 (ANXA2), which was identified in our siRNA screen, and annexin A6 (ANXA6) (68). ANXA2 and ANXA6 are multifunctional proteins involved in endosomal trafficking, segregation of membrane lipids, and membrane curvature regulation through membrane-cytoskeleton rearrangements (69). Disruption of NPC1 leads to increased concentrations of ANXA2 and ANXA6 in late endosomes in response to cholesterol accumulation (70, 71). It is possible that cholesterol accumulation in cells lacking NPC1 similarly alters the distribution or function of WDR81. Thus, dysfunction of endosomal proteins in NPC1 KO cells might alter potential interactions of μ 1N or the reovirus core with specific lipid microdomains or proteins and inhibit core release.

Genetic screens are useful approaches to identify host factors required for viral replication and provide valuable information about virus-cell interactions (72, 73). However, genetic screens frequently yield long lists of potential candidates, many of which are false-positives. To increase the likelihood of identifying host factors required for reovirus replication, we compared gene lists obtained from independent genome-wide CRISPR/Cas9 and siRNA-based cell-survival screens. Only 28 genes in the CRISPR/Cas9 screens using strains T1L and T3D were identified in the siRNA screen using strain T3SA+, 19 of which are ribosomal genes (Fig. 1B, C). Of the nine non-ribosomal genes, several encode proteins required for reovirus entry, including those

involved in sialic acid biosynthesis and metabolism (*Nans* and *Neu*) (37, 38) and viral disassembly (*CtsI*) (22).

Our findings indicate that NPC1, which was identified in both CRISPR/Cas9 and siRNA screens, is required for efficient release of reovirus cores into the cytoplasm by regulating cholesterol homeostasis. High-resolution studies showing the precise distribution of reovirus virions and cores within endosomes will be required to understand how NPC1 and cholesterol homeostasis regulate core release. These studies will allow us to answer the following new questions: Do cores interact with endosomal membranes in NPC1 KO cells? Does cholesterol impede interactions of cores with membranes? Are other lipids or proteins required for core release? Our ongoing work to answer these questions will clarify the functional elements of the reovirus entry pathway and lead to new approaches to block the entry of viruses that depend on tightly regulated cholesterol distribution in the endocytic pathway.

MATERIALS AND METHODS

Cells and viruses

HBMECs were cultured in growth medium (RPMI 1640 (Gibco) supplemented to contain 10% fetal bovine serum (FBS; VWR 97068-085), 10% Nu Serum (Corning), 1% MEM-vitamins (Corning), 1% sodium pyruvate (Gibco), 1% MEM non-essential amino acids (Gibco), 1% L-glutamine (Gibco), 1% penicillin/streptomycin (Gibco), and 0.1% amphotericin B (Sigma) or infection medium (growth medium containing 2% FBS). BV2 mouse microglial cells were cultured in BV2 maintenance medium (DMEM supplemented to contain 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, and 1% sodium bicarbonate) or selection medium (maintenance media supplemented with 4 µg/ml blasticidin (Thermo Fisher) and 2.5 µg/ml puromycin (Sigma-Aldrich)). HeLa cells were cultured in Dulbecco modified Eagle medium (Gibco) supplemented to contain 10% FBS, minimal essential medium nonessential amino acid solution (Gibco), 0.11 mg/mL of sodium pyruvate (Gibco), and 1% penicillin/ streptomycin, and 0.1% amphotericin B (Sigma). Spinner-adapted L929 cells (originally obtained from the Bernard Fields laboratory; ATCC CCL-1) were grown in either suspension or monolayers in Joklik's modified Eagle's minimal essential medium (US Biological; M3867) supplemented to contain 5% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.1% amphotericin B.

Reovirus strains T1L, T3D, T3SA+, and T1L M1-P208S, were prepared from laboratory stocks by plaque purification followed by 3 to 4 passages in L929 cells. T3SA+ contains nine genes from T1L and the S1 gene from T3C44-MA (36). T1L M1-P208S contains a point mutation in the M1 gene that causes viral factories to have a globular morphology similar to the morphology of factories formed by reovirus T3D (43) and can be readily scored for infection. Virions were purified from infected L929 cell

lysates using cesium chloride gradient centrifugation as described (74). Viral titers were determined by plaque assay using L929 cells (75) and expressed as plaque forming units per ml (PFU/ml). Reovirus particle concentration was estimated by spectral absorbance of purified virions at 260 nm (optical density at 260 nm [OD₂₆₀] of 1 = 2.1x10¹² particles/ml) (76).

Fluorescent reovirus particles were prepared by diluting 6 × 10¹² reovirus particles/ml in 50 mM sodium bicarbonate buffer and incubating with 20 μM Alexa Fluor™ 647 NHS Ester (Succinimidyl Ester) (Invitrogen, A37573) at room temperature (RT) for 90 min, protected from light (77). Labeled virions were dialyzed at 4°C overnight with 2-3 buffer exchanges to remove unreacted dye.

ISVPs were prepared by incubating 2 × 10¹² purified reovirus particles with 200 μg/mL chymotrypsin (Sigma, C3142) at 37°C for 60 min (23). Digestion was terminated by the addition of PMSF to a final concentration of 2 mM. Virion-to-ISVP conversion was confirmed by SDS-PAGE and colloidal blue staining to assess the loss of σ3 and cleavage of μ1C to δ.

Antibodies and dyes

Primary antibodies used for indirect immunofluorescence include anti-CD63 (1:250) (ThermoFisher, #10628D), reovirus-specific polyclonal rabbit antiserum (1:1000) (78), and T1L core-specific rabbit antiserum (1:250) provided by Max Nibert (79). Alexa Fluor conjugated secondary antibodies (Thermo Fisher, #A11034, #A11030) were used to visualize antigen. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, D3571). Primary antibodies used for immunoblotting include reovirus-specific polyclonal rabbit antiserum, NPC1-specific polyclonal rabbit antiserum (Abcam, 134113), and mouse GAPDH monoclonal antibody for protein loading controls (Sigma,

482 G8795). Anti-mouse IRDye680RD and anti-rabbit IRDye800CW (Licor) secondary
483 antibodies were used.

484

485 **CRISPR Screen**

486 The screen was conducted and transduction validated as described (80). BV2 cells
487 were transduced with pXPR_101 lentivirus encoding Cas9 (Addgene; 52962) and
488 propagated for 11 days with BV2 Maintenance Medium supplemented to contain
489 blasticidin. These parental BV2 or BV2-Cas9 cells were transduced for 2 days with
490 pXPR_011 expressing eGFP (Addgene; 59702) and a short guide RNA (sgRNA)
491 targeting eGFP at a multiplicity of infection (MOI) of less than 1 PFU/cell. Cells were
492 selected for 5 days with BV2 selection medium. The frequency of eGFP-expressing
493 cells was quantified by flow cytometry.

494 The murine Asiago sgRNA CRISPR library contains six independent genome-
495 wide pools, in which each pool contains unique sgRNAs targeting 20,077 mouse genes.
496 Four pools of the Asiago library were transduced into 5×10^7 BV2 cells at an MOI of 0.2
497 PFU/cell to establish four BV2 libraries. Two days post-transduction, cells were
498 transferred to BV2 Selection Medium and propagated for 5 additional days. For each
499 experimental condition, 10^7 BV2 library cells expressing Cas9 and sgRNAs were
500 seeded in duplicate into T175 tissue culture flasks (Greiner Bio-One). Cells were
501 inoculated with Opti-MEM supplemented to contain PBS (mock) or reovirus strains T1L
502 or T3D at an MOI of 100 PFU/cell. Cells were incubated at RT for 1 h, followed by the
503 addition of 20 mL of DMEM supplemented to contain 10% FBS, 1%
504 penicillin/streptomycin, 1% sodium pyruvate, and 1% sodium bicarbonate. After 2 days
505 post-inoculation (dpi) (mock) or 9 dpi (T1L or T3D conditions), cells were harvested and

genomic DNA (gDNA) was isolated from surviving cells using a QIAmp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions.

CRISPR screen sequencing and analysis

Illumina sequencing and STARS analyses were conducted as described (81). The gDNA was aliquoted into a 96-well plate (Greiner Bio-One) with up to 10 µg gDNA in 50 µL of total volume per well. A polymerase chain reaction (PCR) master mix containing ExTaq DNA polymerase (Clontech), ExTaq buffer (Clontech), dNTPs, P5 stagger primer, and water was prepared. PCR master mix (40 µL) and 10 µL of a barcoded primer were added to each well containing gDNA. Samples were amplified using the following protocol: 95°C for 1 min, followed by 28 cycles of 94°C for 50 s, 52.5°C for 30 s, and 72°C for 30 s, and ending with a final 72°C extension for 10 min. PCR product was purified using Agencourt AMPure XP SPRI beads (Beckman Coulter) according to the manufacturer's instructions. Samples were sequenced using a HiSeq 2000 (Illumina). Following deconvolution of the barcodes in the P7 primer, sgRNA sequences were mapped to a reference file of sgRNAs from the Asiago library. To account for the varying number of reads per condition, read counts per sgRNA were normalized to 10⁷ total reads per sample. Normalized values were then log-2 transformed. sgRNAs that were not detected were arbitrarily assigned a read count of 1. sgRNA frequencies were analyzed using STARS software to produce a rank ordered score for each gene. This score correlated with the sgRNA candidates that were above 10% of the total sequenced sgRNAs. Genes scoring above this threshold in either of the two independent subpools and in at least two of the four independent genome-wide pools were assigned a STAR score. In addition to the STAR score, screen results were

compared using false discovery rate (FDR) analyses to monitor gene-specific signal versus background noise. Statistical values of independent replicates were averaged.

Whole genome siRNA screen and analysis

The whole genome siRNA screen was conducted as described (35) using HeLa S3 cells and the Dharmacon ON-TARGETplus® SMARTpool® human siRNA library (Thermo Scientific) and strain T3SA+.

Production of NPC1 KO and KO+ cell lines

HBMEC single-cell clones with ablation of the *NPC1* gene were engineered using CRISPR/Cas9-mediated gene editing as described (82) using an NPC1-specific gRNA (5' GGCCTTGTCATTACTTGAGGGGG 3', targeting nucleotides 768-790 of the human NPC1 mRNA). Single-cell clones were screened for the loss of NPC1 function by filipin III staining (82). Genotype of the selected NPC1 KO clones was confirmed by Sanger sequencing followed by amplification of the genomic DNA sequences flanking the gRNA target site using forward (5' TCATAAACACACCAAACCTTGGAATC 3') and reverse (5' TCCTGCGGCAGAGGTTTTTC 3') primers. Sequences of the NPC1 alleles were deconvoluted using CRISP-ID (83). To confirm the specificity of *Npc1* knockout, cells of a single clone were transduced with a retrovirus vector (pBabe-Puro) expressing human NPC1 as described (47).

Indirect immunofluorescence staining

Cells were fixed with 4% paraformaldehyde (PFA, Electron Microscopy Sciences, 15712-s) in PBS^{-/-} at RT for 20 min, washed three times with PBS^{-/-}, and permeabilized and blocked with 0.1% Triton X-100 and 2% FBS in PBS^{-/-} at RT for 20 min. Cells were

incubated sequentially with primary antibody, Alexa Fluor-conjugated secondary antibody, and DAPI diluted in PBS^{-/-} containing 0.1% Triton X-100 and 2% FBS at RT for 30 to 60 min. For cholesterol labeling, fixed and permeabilized cells were incubated with 50 µg/ml filipin III (Sigma, SAE0088) diluted in PBS^{-/-} for 30 min. Coverslips were mounted using Prolong-gold (Molecular Probes). Confocal images were captured using a Leica-SP8 laser scanning confocal microscope equipped with an HCX PL APO 63X/1.4 N.A oil objective and processed using Fiji/ImageJ software.

SDS-PAGE and Immunoblotting

Cells harvested for protein extraction were lysed in Radioimmunoprecipitation Assay buffer (*RIPA buffer*, Thermo Fisher) supplemented with 1X protease inhibitors (Thermo Fisher). Protein concentration was quantified by Bradford assay (Bio-Rad) following the manufacturer's protocol. Samples for SDS-PAGE were diluted in 5X Laemmli sample buffer (Bio-Rad) containing 10% β-mercaptoethanol and incubated at 95°C for 10 min. Samples for detection of NPC1 were incubated at 70°C for 10 min to prevent aggregation. Equal amounts of protein were electrophoresed in 10% or 4-20% Mini-Protean TGX gels (Bio-Rad). Following electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad) for immunoblotting. Nitrocellulose membranes were incubated with 5% nonfat milk in TBS (50 mM Tris-HCl, pH 7.6; 150 mM NaCl) with 0.1% Tween 20 (TBS-T) and sequentially incubated with primary and secondary antibodies diluted in TBS-T at RT for 1 h. Immunoblot images were captured using an Odyssey CLx imaging system (Li-Cor) and protein bands were quantified using the Image Studio Lite software. Protein expression levels were normalized to GAPDH loading controls.

Quantification of reovirus infectivity

In experiments comparing infectivity of reovirus in KO, KO+, and WT HBMECs, cells were adsorbed with 10,000 reovirus virions or 100 ISVPs diluted in Opti-MEM (Invitrogen) at 37°C for 1 h. Following adsorption, the inoculum was removed, and cells were incubated in infection medium for 18 h before fixing in ice-cold methanol. In experiments comparing reovirus infectivity in the presence or absence of H β CD, cells were treated with 1 mM H β CD or PBS for 24 h prior to adsorption with reovirus. Following adsorption, fresh 1 mM H β CD was added to the medium for 18 h before fixing in ice-cold methanol. Fixed cells were washed with PBS^{-/-}, blocked with 1% bovine serum albumin (BSA), and incubated sequentially with reovirus-specific polyclonal rabbit antiserum, Alexa Fluor 488-conjugated anti-rabbit antibody, and DAPI in PBS^{-/-} containing 0.5% Triton X-100. Cells were imaged using a Lionheart FX automated imager (BioTek) equipped with a 20X air objective, taking four fields-of-view from duplicate samples. Images were processed and signals quantified using Gen5+ software (BioTek).

Viral binding

KO, KO+, and WT HBMECs were detached from tissue-culture plates using CellStripper dissociation reagent (Corning), quenched with HBMEC medium, and washed with PBS^{-/-}. Cells were resuspended in PBS^{-/-} at 10⁶ cells/ml and adsorbed with 10,000 Alexa Fluor 647-labeled reovirus virions/cell at 4°C for 1 h with agitation. After binding, cells were washed twice with PBS^{-/-} and fixed with 1% paraformaldehyde (PFA) supplemented with propidium iodide to determine cell viability. Cells were analyzed using an LSRII flow cytometer (BD Bioscience). Results were quantified using FlowJo V10 software.

605

606 **Live microscopy of reovirus internalization**

607 KO, KO+, and WT HBMECs were plated on glass-bottom p35 plates and adsorbed with
608 10,000 Alexa 647-labeled reovirus virions/cell at 4°C for 45 min to synchronize
609 infection. The inoculum was removed and replaced with fresh Opti-MEM without
610 phenol-red medium supplemented with 2% FBS. Reovirus transport was imaged using
611 a Leica DMI6000B fluorescence microscope with an HCX PL APO 63X/1.30 Gly
612 objective. Fluorescence and brightfield images were collected from 0 to 36 min post
613 adsorption every ~ 25 sec.

614

615 **Tracking of reovirus transport**

616 Automated tracking of fluorescent reovirus particles in time-lapse images was
617 conducted using Icy bioimage analysis software. Regions of interest (ROI)
618 corresponding to the cell periphery were selected for tracking analysis using the Spot
619 Detector plugin (84). The scale of the object (reovirus virions) to be analyzed was set at
620 a size of ~7 pixels per spot, and the threshold sensitivity was set at 100. Parameters
621 describing transport dynamics were considered as both diffusive and directed for
622 running tracking analysis. Results are presented in colored time-dependent tracks.

623

624 **Quantification of reovirus cores**

625 KO, KO+, and WT HBMECs were adsorbed with 10,000 Alexa Fluor 647-labeled
626 reovirus virions at 37°C for 45 min. The inoculum was removed, and the cells were
627 incubated in infection medium containing 100 µg/ml of cycloheximide for 8 h. After
628 fixation, cells were permeabilized and stained with T1L core-specific rabbit polyclonal
629 serum and anti-CD63 antibody. Confocal images were captured using a Leica-SP8

laser scanning confocal microscope equipped with an HCX PL APO 63X/1.4 N.A oil objective and processed using Fiji/ImageJ software. Colocalization of fluorescent reovirus virions (cyan puncta), reovirus cores (green puncta), and late endosomes (red puncta) was analyzed to differentiate infecting virions from cores released into the cytoplasm.

RNA extraction and purification

Cells were lysed using TRIzol reagent (Invitrogen). RNA was extracted with chloroform and purified using a PureLink RNA minikit (Invitrogen) with DNase treatment according to the manufacturer's instructions.

S4 quantitative RT-PCR

Total S4 RNA was quantified using qScript XLT one-step RT-qPCR ToughMix, Low ROX (Quanta Bioscience) and T3D_S4_qPCR primers (Forward: GAAGCATTTGCCTCACCATAG, Reverse: GATCTGTCCAACCTTGAGTGTATTG) according to the manufacturer's instructions. The following RT-qPCR cycling protocol was used: cDNA synthesis (50°C for 10 min), initial denaturation (95°C for 1 min), and 40 PCR cycles (95°C for 10 s followed by a data collection step at 60°C for 1 min). S4 cDNA was detected using a fluorogenic probe (5'-FAM [fluorescent fluorescein]-AGCGCGCAAGAGGGATGGGA-BHQ [black hole quencher]-1-3'; Biosearch Technologies).

Statistical analysis

653 All data were analyzed using Graphpad Prism 8. Figure legends specify the number of
654 experimental repeats and the statistical test applied for each analysis. Differences were
655 considered statistically significant when P values were less than 0.05.

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667

668 **Competing interests**

669 The authors have declared that no competing interests exist.

670

671 **Author contributions**

672 **Conceived and designed experiments:** POG, GT, CR, and TSD. **Conducted**
673 **experiments:** POG, GT, RKJ, BAM, RCO, and CBW. **Analyzed data:** POG and GT.
674 **Contributed reagents/materials/analysis tools:** RKJ, RCO, CBW, and KC. **Wrote**
675 **original draft:** POG, GT, and TSD. **Reviewed and edited paper:** POG, GT, RKJ, RT,
676 IF, BAM, RCO, CBW, PAB, JS, KC, CR, and TSD.

REFERENCES

1. Mercer J, Helenius A. Virus entry by macropinocytosis. *Nat Cell Biol.* 2009;11(5):510-20.
2. Mercer J, Schelhaas M, Helenius A. Virus entry by endocytosis. *Annu Rev Biochem.* 2010;79:803-33.
3. Yamauchi Y, Helenius A. Virus entry at a glance. *J Cell Sci.* 2013;126(Pt 6):1289-95.
4. Smith AE, Helenius A. How viruses enter animal cells. *Science.* 2004;304(5668):237-42.
5. Tsai B. Penetration of nonenveloped viruses into the cytoplasm. *Annu Rev Cell Develop Biol.* 2007;23:23-43.
6. Staring J, Raaben M, Brummelkamp TR. Viral escape from endosomes and host detection at a glance. *J Cell Sci.* 2018;131(15).
7. Harrison SC. Viral membrane fusion. *Virology.* 2015;479-480:498-507.
8. White JM, Whittaker GR. Fusion of enveloped viruses in endosomes. *Traffic.* 2016;17(6):593-614.
9. Sieczkarski SB, Whittaker GR. Viral entry. *Curr Top Microbiol Immunol.* 2005;285:1-23.
10. Plemper RK. Cell entry of enveloped viruses. *Curr Opin Virol.* 2011;1(2):92-100.
11. Stewart PL, Dermody TS, Nemerow GR. Structural basis of nonenveloped virus cell entry. *Adv Protein Chem.* 2003;64:455-91.
12. Kumar CS, Dey D, Ghosh S, Banerjee M. Breach: Host membrane penetration and entry by nonenveloped viruses. *Trends Microbiol.* 2018;26(6):525-37.
13. Banerjee M, Johnson JE. Activation, exposure and penetration of virally encoded, membrane-active polypeptides during non-enveloped virus entry. *Curr Protein Pept Sci.* 2008;9(1):16-27.
14. Bouziat R, Hinterleitner R, Brown JJ, Stencel-Baerenwald JE, Ikizler M, Mayassi T, et al. Reovirus infection triggers inflammatory responses to dietary antigens and development of celiac disease. *Science.* 2017;356(6333):44-50.
15. Dryden KA, Wang G, Yeager M, Nibert ML, Coombs KM, Furlong DB, et al. Early steps in reovirus infection are associated with dramatic changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction. *J Cell Biol.* 1993;122(5):1023-41.
16. Zhang X, Ji Y, Zhang L, Harrison SC, Marinescu DC, Nibert ML, et al. Features of reovirus outer capsid protein m1 revealed by electron cryomicroscopy and image reconstruction of the virion at 7.0 Å resolution. *Structure.* 2005;13(10):1545-57.

- 713 17. Dermody TS, Parker JS, Sherry B. Orthoreoviruses. In: Knipe DM, Howley PM,
714 editors. Fields Virology. 2. 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013. p.
715 1304-46.
- 716 18. Gummersheimer SL, Snyder AJ, Danthi P. Control of capsid transformations
717 during reovirus entry. *Viruses*. 2021;13(2):153.
- 718 19. Roth AN, Aravamudhan P, Fernández de Castro I, Tenorio R, Risco C, Dermody
719 TS. Ins and outs of reovirus: vesicular trafficking in viral entry and egress. *Trends*
720 *Microbiol*. 2021;29(4):363-75.
- 721 20. Borsa J, Sargent MD, Lievaart PA, Copps TP. Reovirus: evidence for a second
722 step in the intracellular uncoating and transcriptase activation process. *Virology*.
723 1981;111(1):191-200.
- 724 21. Sturzenbecker LJ, Nibert ML, Furlong DB, Fields BN. Intracellular digestion of
725 reovirus particles requires a low pH and is an essential step in the viral infectious cycle.
726 *J Virol* 1987;61(8):2351-61.
- 727 22. Ebert DH, Deussing J, Peters C, Dermody TS. Cathepsin L and cathepsin B
728 mediate reovirus disassembly in murine fibroblast cells. *J Biol Chem*.
729 2002;277(27):24609-17.
- 730 23. Baer GS, Dermody TS. Mutations in reovirus outer-capsid protein s3 selected
731 during persistent infections of L cells confer resistance to protease inhibitor E64. *J Virol*.
732 1997;71:4921-8.
- 733 24. Silverstein SC, Astell C, Levin DH, Schonberg M, Acs G. The mechanism of
734 reovirus uncoating and gene activation in vivo. *Virology*. 1972;47(3):797-806.
- 735 25. Snyder AJ, Danthi P. Lipid membranes facilitate conformational changes
736 required for reovirus cell entry. *J Virol*. 2015;90(5):2628-38.
- 737 26. Snyder AJ, Danthi P. Lipids cooperate with the reovirus membrane penetration
738 peptide to facilitate particle uncoating. *J Biol Chem*. 2016;291(52):26773-85.
- 739 27. Odegard AL, Chandran K, Zhang X, Parker JS, Baker TS, Nibert ML. Putative
740 autocleavage of outer capsid protein m1, allowing release of myristoylated peptide m1N
741 during particle uncoating, is critical for cell entry by reovirus. *J Virol*. 2004;78(16):8732-
742 45.
- 743 28. Agosto MA, Ivanovic T, Nibert ML. Mammalian reovirus, a nonfusogenic
744 nonenveloped virus, forms size-selective pores in a model membrane. *Proc Natl Acad*
745 *Sci U S A*. 2006;103(44):16496-501.
- 746 29. Ivanovic T, Agosto MA, Zhang L, Chandran K, Harrison SC, Nibert ML. Peptides
747 released from reovirus outer capsid form membrane pores that recruit virus particles.
748 *EMBO J*. 2008;27(8):1289-98.
- 749 30. Chandran K, Farsetta DL, Nibert ML. Strategy for nonenveloped virus entry: a
750 hydrophobic conformer of the reovirus membrane penetration protein m1 mediates
751 membrane disruption. *J Virol*. 2002;76(19):9920-33.

- 752 31. Zhang L, Agosto MA, Ivanovic T, King DS, Nibert ML, Harrison SC.
753 Requirements for the formation of membrane pores by the reovirus myristoylated
754 micro1N peptide. *J Virol.* 2009;83(14):7004-14.
- 755 32. Carstea ED, Morris JA, Coleman KG, Loftus SK, Zhang D, Cummings C, et al.
756 Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis.
757 *Science.* 1997;277(5323):228-31.
- 758 33. Ioannou YA. The structure and function of the Niemann-Pick C1 protein. *Mol*
759 *Genet Metab.* 2000;71(1-2):175-81.
- 760 34. Kwon HJ, Abi-Mosleh L, Wang ML, Deisenhofer J, Goldstein JL, Brown MS, et
761 al. Structure of N-terminal domain of NPC1 reveals distinct subdomains for binding and
762 transfer of cholesterol. *Cell.* 2009;137(7):1213-24.
- 763 35. Konopka-Anstadt JL, Mainou BA, Sutherland DM, Sekine Y, Strittmatter SM,
764 Dermody TS. The Nogo receptor NgR1 mediates infection by mammalian reovirus. *Cell*
765 *Host Microbe.* 2014;15(6):681-91.
- 766 36. Barton ES, Connolly JL, Forrest JC, Chappell JD, Dermody TS. Utilization of
767 sialic acid as a coreceptor enhances reovirus attachment by multistep adhesion
768 strengthening. *J Biol Chem.* 2001;276(3):2200-11.
- 769 37. Urbanek K, Sutherland DM, Orchard RC, Wilen CB, Knowlton JJ, Aravamudhan
770 P, et al. Cytidine monophosphate N-acetylneuraminic acid synthetase and solute carrier
771 family 35 member A1 are required for reovirus binding and infection. *J Virol.* 2020;95(2).
- 772 38. Gentsch JR, Pacitti AF. Effect of neuraminidase treatment of cells and effect of
773 soluble glycoproteins on type 3 reovirus attachment to murine L cells. *J Virol.*
774 1985;56(2):356-64.
- 775 39. Avula K, Singh B, Kumar PV, Syed GH. Role of lipid transfer proteins (LTPs) in
776 the viral life cycle. *Front Microbiol.* 2021;12:673509.
- 777 40. Lai CM, Mainou BA, Kim KS, Dermody TS. Directional release of reovirus from
778 the apical surface of polarized endothelial cells. *mBio.* 2013;4(2):e00049-13.
- 779 41. Fu J, Li L, Huo D, Zhi S, Yang R, Yang B, et al. Astrocyte-derived TGFβ1
780 facilitates blood-brain barrier function via non-canonical hedgehog signaling in brain
781 microvascular endothelial cells. *Brain Sci.* 2021;11(1).
- 782 42. Borsa J, Copps TP, Sargent MD, Long DG, Chapman JD. New intermediate
783 subviral particles in the in vitro uncoating of reovirus virions by chymotrypsin. *J Virol.*
784 1973;11(4):552-64.
- 785 43. Parker JS, Broering TJ, Kim J, Higgins DE, Nibert ML. Reovirus core protein m2
786 determines the filamentous morphology of viral inclusion bodies by interacting with and
787 stabilizing microtubules. *J Virol.* 2002;76(9):4483-96.
- 788 44. Aravamudhan P, Raghunathan K, Konopka-Anstadt J, Pathak A, Sutherland DM,
789 Carter BD, et al. Reovirus uses macropinocytosis-mediated entry and fast axonal
790 transport to infect neurons. *PLoS Pathog.* 2020;16(2):e1008380.

- 791 45. Elrick MJ, Yu T, Chung C, Lieberman AP. Impaired proteolysis underlies
792 autophagic dysfunction in Niemann-Pick type C disease. *Hum Mol Genet.*
793 2012;21(22):4876-87.
- 794 46. Carette JE, Raaben M, Wong AC, Herbert AS, Obernosterer G, Mulherkar N, et
795 al. Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature.*
796 2011;477(7364):340-3.
- 797 47. Miller EH, Obernosterer G, Raaben M, Herbert AS, Deffieu MS, Krishnan A, et al.
798 Ebola virus entry requires the host-programmed recognition of an intracellular receptor.
799 *EMBO J.* 2012;31(8):1947-60.
- 800 48. Rosenbaum AI, Zhang G, Warren JD, Maxfield FR. Endocytosis of beta-
801 cyclodextrins is responsible for cholesterol reduction in Niemann-Pick type C mutant
802 cells. *Proc Natl Acad Sci U S A.* 2010;107(12):5477-82.
- 803 49. Peake KB, Vance JE. Normalization of cholesterol homeostasis by 2-
804 hydroxypropyl- β -cyclodextrin in neurons and glia from Niemann-Pick C1 (NPC1)-
805 deficient mice. *J Biol Chem.* 2012;287(12):9290-8.
- 806 50. Liu B, Turley SD, Burns DK, Miller AM, Repa JJ, Dietschy JM. Reversal of
807 defective lysosomal transport in NPC disease ameliorates liver dysfunction and
808 neurodegeneration in the npc1-/- mouse. *Proc Natl Acad Sci U S A.* 2009;106(7):2377-
809 82.
- 810 51. Ory DS, Ottinger EA, Farhat NY, King KA, Jiang X, Weissfeld L, et al. Intrathecal
811 2-hydroxypropyl- β -cyclodextrin decreases neurological disease progression in
812 Niemann-Pick disease, type C1: a non-randomised, open-label, phase 1-2 trial. *Lancet.*
813 2017;390(10104):1758-68.
- 814 52. Poh MK, Shui G, Xie X, Shi PY, Wenk MR, Gu F. U18666A, an intra-cellular
815 cholesterol transport inhibitor, inhibits dengue virus entry and replication. *Antiviral Res.*
816 2012;93(1):191-8.
- 817 53. Cuesta-Geijo M, Chiappi M, Galindo I, Barrado-Gil L, Muñoz-Moreno R,
818 Carrascosa JL, et al. Cholesterol flux is required for endosomal progression of African
819 swine fever virions during the initial establishment of infection. *J Virol.* 2016;90(3):1534-
820 43.
- 821 54. Elgner F, Ren H, Medvedev R, Ploen D, Himmelsbach K, Boller K, et al. The
822 intracellular cholesterol transport inhibitor U18666A inhibits the exosome-dependent
823 release of mature hepatitis C virus. *J Virol.* 2016;90(24):11181-96.
- 824 55. Yin X, Ambardekar C, Lu Y, Feng Z. Distinct entry mechanisms for
825 nonenveloped and quasi-enveloped hepatitis E viruses. *J Virol.* 2016;90(8):4232-42.
- 826 56. Rivera-Serrano EE, González-López O, Das A, Lemon SM. Cellular entry and
827 uncoating of naked and quasi-enveloped human hepatoviruses. *eLife.* 2019;8.
- 828 57. Sobo K, Le Blanc I, Luyet PP, Fivaz M, Ferguson C, Parton RG, et al. Late
829 endosomal cholesterol accumulation leads to impaired intra-endosomal trafficking. *PloS*
830 *one.* 2007;2(9):e851.
- 831 58. Vanier MT. Niemann-Pick disease type C. *Orphanet J Rare Dis.* 2010;5:16.

59. Koller D, Lohner K. The role of spontaneous lipid curvature in the interaction of interfacially active peptides with membranes. *Biochim Biophys Acta*. 2014;1838(9):2250-9.
60. Maier O, Galan DL, Wodrich H, Wiethoff CM. An N-terminal domain of adenovirus protein VI fragments membranes by inducing positive membrane curvature. *Virology*. 2010;402(1):11-9.
61. Guo X, Steinkühler J, Marin M, Li X, Lu W, Dimova R, et al. Interferon-induced transmembrane protein 3 blocks fusion of diverse enveloped viruses by altering mechanical properties of cell membranes. *ACS Nano*. 2021;15(5):8155-70.
62. Anafu AA, Bowen CH, Chin CR, Brass AL, Holm GH. Interferon-inducible transmembrane protein 3 (IFITM3) restricts reovirus cell entry. *J Biol Chem*. 2013;288(24):17261-71.
63. Moyer CL, Wiethoff CM, Maier O, Smith JG, Nemerow GR. Functional genetic and biophysical analyses of membrane disruption by human adenovirus. *J Virol*. 2011;85(6):2631-41.
64. Patel A, Mohl BP, Roy P. Entry of Bluetongue virus capsid requires the late endosome-specific lipid lysobisphosphatidic acid. *J Biol Chem*. 2016;291(23):12408-19.
65. Silva-Ayala D, López T, Gutiérrez M, Perrimon N, López S, Arias CF. Genome-wide RNAi screen reveals a role for the ESCRT complex in rotavirus cell entry. *Proc Natl Acad Sci U S A*. 2013;110(25):10270-5.
66. Snyder A, Abad A, Danthi, P. A CRISPR-Cas9 screen reveals a role for WD repeat-containing protein (WDR81) in the entry of late penetrating viruses. *bioRxiv preprint*, doi:<https://doi.org/10.1101/2021.09.26.461887>, 2021.
67. Liu K, Jian Y, Sun X, Yang C, Gao Z, Zhang Z, et al. Negative regulation of phosphatidylinositol 3-phosphate levels in early-to-late endosome conversion. *J Cell Biol*. 2016;212(2):181-98.
68. Wheeler S, Schmid R, Sillence DJ. Lipid-protein interactions in Niemann-Pick type C Disease: Insights from molecular modeling. *Int J Mol Sci*. 2019;20(3).
69. Rentero C, Blanco-Munoz P, Meneses-Salas E, Grewal T, Enrich C. Annexins-coordinators of cholesterol homeostasis in endocytic pathways. *Int J Mol Sci*. 2018;19(5):E1444.
70. de Diego I, Schwartz F, Siegfried H, Dauterstedt P, Heeren J, Beisiegel U, et al. Cholesterol modulates the membrane binding and intracellular distribution of annexin 6. *J Biol Chem*. 2002;277(35):32187-94.
71. Mayran N, Parton RG, Gruenberg J. Annexin II regulates multivesicular endosome biogenesis in the degradation pathway of animal cells. *EMBO J*. 2003;22(13):3242-53.
72. Hirsch AJ. The use of RNAi-based screens to identify host proteins involved in viral replication. *Future Microbiol*. 2010;5(2):303-11.

871 73. Puschnik AS, Majzoub K, Ooi YS, Carette JE. A CRISPR toolbox to study virus-
872 host interactions. *Nat Rev Microbiol.* 2017;15(6):351-64.

873 74. Furlong DB, Nibert ML, Fields BN. Sigma 1 protein of mammalian reoviruses
874 extends from the surfaces of viral particles. *J Virol.* 1988;62(1):246-56.

875 75. Virgin HW, Bassel-Duby R, Fields BN, Tyler KL. Antibody protects against lethal
876 infection with the neurally spreading reovirus type 3 (Dearing). *J Virol.*
877 1988;62(12):4594-604.

878 76. Smith RE, Zweerink HJ, Joklik WK. Polypeptide components of virions, top
879 component and cores of reovirus type 3. *Virology.* 1969;39(4):791-810.

880 77. Fecek RJ, Busch R, Lin H, Pal K, Cunningham CA, Cuff CF. Production of Alexa
881 Fluor 488-labeled reovirus and characterization of target cell binding, competence, and
882 immunogenicity of labeled virions. *J Immunol Methods.* 2006;314(1-2):30-7.

883 78. Wetzel JD, Chappell JD, Fogo AB, Dermody TS. Efficiency of viral entry
884 determines the capacity of murine erythroleukemia cells to support persistent infections
885 by mammalian reoviruses. *J Virol.* 1997;71(1):299-306.

886 79. Chandran K, Walker SB, Chen Y, Contreras CM, Schiff LA, Baker TS, et al. In
887 vitro recoating of reovirus cores with baculovirus-expressed outer-capsid proteins $\mu 1$
888 and $\sigma 3$. *J Virol.* 1999;73(5):3941-50.

889 80. Orchard RC, Wilen CB, Doench JG, Baldrige MT, McCune BT, Lee YC, et al.
890 Discovery of a proteinaceous cellular receptor for a norovirus. *Science.*
891 2016;353(6302):933-6.

892 81. Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, et al.
893 Optimized sgRNA design to maximize activity and minimize off-target effects of
894 CRISPR-Cas9. *Nat Biotechnol.* 2016;34(2):184-91.

895 82. Spence JS, Krause TB, Mittler E, Jangra RK, Chandran K. Direct visualization of
896 ebola virus fusion triggering in the endocytic pathway. *mBio.* 2016;7(1):e01857-15.

897 83. Dehairs J, Talebi A, Cherifi Y, Swinnen JV. CRISP-ID: decoding CRISPR
898 mediated indels by Sanger sequencing. *Sci Rep.* 2016;6:28973.

899 84. Olivio-Marin JC. Extraction of spots in biological images using multi-scale
900 products. *Pattern Recognition.* 2002;35(9):1989-96.

FIGURE LEGENDS

Fig. 1. CRISPR and siRNA screens identify NPC1 as a cellular factor required for reovirus infection. (A) The top 20 candidates from the CRISPR screen using reovirus strains T1L and T3D are ranked by their STAR scores. Heat map indicates STAR values. (B) Genes from the siRNA screen using reovirus strain T3SA+ common to the CRISPR screen using T1L and T3D, excluding ribosomal genes. Heat map indicates z-score values. (C) Venn diagram of genes from the CRISPR screens using T1L and T3D and the siRNA screen using T3SA+. (D) Molecular function pathways using Gene Ontology to analyze genes from the CRISPR screen common to T1L and T3D. (E) KEGG pathways identified for the CRISPR screen using T1L (red) and T3D (blue) and siRNA screen using T3SA+ (light blue).

Fig. 2. Viral infectivity and titers following adsorption by reovirus virions and ISVPs. (A, B) WT, KO, and KO+ HBMECs were adsorbed with reovirus (A) virions or (B) ISVPs at MOIs of 10,000 or 100 particles/cell, respectively, and fixed at 18 h post-adsorption. The percentage of infected cells was determined by enumerating reovirus-infected cells following immunostaining with a reovirus-specific antiserum. (C-F) WT, KO, and KO+ cells were adsorbed with reovirus (C, E) virions at an MOI of 1 PFU/cell or (D, F) ISVPs at an MOI of 5 particles/cell. Viral titers in cell-culture supernatants and lysates were determined by plaque assay at 0, 24, and 48 h post-adsorption. The results are presented as the mean of three independent experiments. Error bars indicated standard deviation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, as determined by t-test.

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Fig. 4. Cytosolic entry of reovirus cores. (A) WT, KO, and KO+ HBMECs were adsorbed with Alexa 647 labeled-reovirus virions at an MOI of 10,000 particles/cell at 37°C for 45 min and fixed with 4% PFA at 8 h post-adsorption. Cells were stained with DAPI, a CD-63-specific antibody to label endosomes, and an antiserum specific for reovirus cores, and imaged using confocal microscopy. Representative confocal micrographs are shown. (B) Colocalization of reovirus, cores, and endosomes was analyzed using the JaCoP plugin function from ImageJ. The results are presented as the mean colocalization (quantified by Manders coefficient) of ~ 50 cells from three

independent experiments. Error bars indicate standard deviation. **, $P < 0.01$; ***, $P < 0.001$, as determined by two-tailed unpaired t-test.

Fig. 5. Synthesis of nascent RNA is reduced in NPC1 KO HBMECs. WT, KO, and KO+ HBMECs were adsorbed with reovirus virions at an MOI of 1 PFU/cell at 37°C for 1 h, lysed at the intervals post-adsorption shown, and assayed for positive-sense reovirus s4 RNA by RT-qPCR. The results are presented as the mean number of copies of reovirus s4 RNA by qPCR from two independent experiments. Error bars indicate standard errors of the mean. **, $P < 0.01$; ***, $P < 0.001$, as determined by t-test.

Fig. 6. H β CD treatment restores reovirus infection of NPC1 KO HBMECs. WT, KO, and KO+ HBMECs were pretreated with 1 mM H β CD or PBS for 24 h, adsorbed with reovirus virions or ISVPs at MOIs of 10,000 or 100 particles/cell, respectively, and fixed at 18 h post-adsorption. The percentage of infected cells was determined by enumerating reovirus-infected cells following immunostaining with a reovirus-specific antiserum. The results are presented as the mean of three independent experiments. Error bars indicated standard deviation. ***, $P < 0.001$ as determined by two-tailed unpaired t-test.

SUPPLEMENTAL MATERIALS

FIGURES AND MOVIES

Fig. S1. Effect on cholesterol distribution by disruption of NPC1 expression. (A, B) Lysates of WT, KO, and KO+ HBMECs were subjected to electrophoresis and

immunoblotting using an NPC1 antiserum. GAPDH was used as loading control. A representative immunoblot is shown. The results are presented as the mean of two independent experiments. Error bars indicate standard deviation. Statistical analysis was done by two-tailed unpaired t-test. (C) WT, KO, and KO+ HBMECs were stained with filipin III to detect cholesterol distribution. Representative images are shown. Scale bars, 10 μ m. (D) WT, KO, and KO+ HBMECs were stained with filipin III and an anti-CD63 antibody to detect the subcellular localization of cholesterol. Representative images are shown. Scale bars, 10 μ m.

Fig. S2. Viral infectivity following adsorption by T1L, T3D, and T3SA+ virions. (A, B) WT, KO, and KO+ HBMECs were adsorbed with reovirus virions at MOIs of 10,000 particles/cell, and fixed at 18 h post-adsorption. The percentage of infected cells was determined by enumerating reovirus-infected cells following immunostaining with a reovirus-specific antiserum. Error bars indicated standard deviation. **, $P < 0.01$; ***, $P < 0.001$, as determined by 2-way ANOVA, Tukey's multiple comparisons test.

Fig. S3. H β CD treatment restores cholesterol efflux in KO cells. (A) WT, KO, and KO+ HBMECs were treated with H β CD at the concentrations shown for 48 h and assessed for viability using the Presto blue cell viability reagent. The results are presented as the mean cell viability of three independent experiments. Error bars indicated standard deviation. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, as determined by two-way ANOVA. (B, C) Cells were treated with 1 mM H β CD or PBS (mock) for 48 h, fixed with 4% PFA, stained with filipin III, and imaged using confocal microscopy. (B) The results are presented as the mean filipin III staining (quantified by MFI) of ~ 50 cells from three independent experiments. Error bars indicate the minimum

1001 and the maximum values. *, $P < 0.05$; ****, $P < 0.0001$, as determined by two-tailed
1002 unpaired t-test. (C) Representative images of cholesterol distribution in H β CD-treated
1003 and mock-treated cells are shown. Scale bars, 10 μ m.

1004

1005 **VIDEO 1, 2, and 3. High-magnification, live-cell microscopy of fluorescent**
1006 **reovirus virion transport in WT, KO, and KO+ HBMECs.** (1) WT, (2) KO, and (3)
1007 KO+ cells were adsorbed with Alexa 647-labeled reovirus virions at an MOI of 10,000
1008 particles/cell at 4°C for 45 min. Fluorescence and brightfield images were captured
1009 every ~ 25 seconds for 36 min.

1010

1011 **VIDEO 4, 5, and 6. Tracking of fluorescent reovirus virions recruited to a**
1012 **perinuclear region following entry.** Trajectories of reovirus virions during
1013 internalization into WT, KO, and KO+ HBMECs from videos 1, 2, and 3 were tracked
1014 with the spot-tracking plugin function of Icy-Bioimage analysis software (84). Cell
1015 contour was defined as a region of interest (ROI), and ~ 7 pixels/spot were monitored.
1016 The colored bar represents the trajectory depending on time, in which each color (from
1017 yellow to red) corresponds to an interval of ~ 7.5 min in the time-lapse videos. Scale
1018 bars, 10 μ m.

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